that statins cause disruption of cholesterol-containing microdomains, this way exerting an additional effect on MHC class II expression. Recently, much attention has been given to lipid rafts as a platform for signal transduction. A limited number of papers show data supporting or rejecting the hypothesis that statins disrupt lipid rafts[8,9], and we look forward to the paper containing these unpublished observations to which the authors refer in this correspondence.

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Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells

To the editor:

Intramyocardial injection of adult bone marrow–derived stem cells has recently been proposed as a potential therapy to repair damaged myocardium after acute infarction[1–4]. The mechanisms underlying their therapeutic effect have not been clearly defined, with an intense debate over differentiation versus fusion[2,5]. In this report, we provide evidence for a novel mechanism that involves the secretion of paracrine cytoprotective factor(s) from the stem cells. We have previously reported that rat bone marrow–derived mesenchymal stem cells (MSCs) overexpressing the survival gene *Akt1* (Akt-MSCs) are superior to control MSCs transduced with green fluorescent protein (GFP-MSCs) for cell therapy of acute myocardial infarction. Specifically, intramyocardial injection of Akt-MSCs prevents ventricular remodeling and restores cardiac function when measured 2 weeks after infarction[6]. We have recent evidence[7] that these effects occur in less than 72 h. Because this early recovery cannot be explained by the occurrence of meaningful regeneration resulting from donor cell cardiomyogenic differentiation, we postulated that it is achieved through protection of the ischemic myocardium by paracrine mediator(s) released *in situ* by the MSCs. To test our hypothesis, we first assessed the effects of conditioned medium from cultured MSCs *in vitro* on adult rat ventricular cardiomyocytes (ARVCs) subjected to hypoxia. Serum-free conditioned medium (α-minimum essential medium, α-MEM) was collected from MSCs after 12 h of exposure either to normoxia or hypoxia. Hypoxic conditions were created by incubating the cells at 37 °C in an airtight Plexiglas chamber with an atmosphere of 5% CO_2 and 95% N_2 and a controlled oxygen level of 0.5%. Initially, the ARVC standard growth medium was replaced with α-MEM control medium, normoxic-conditioned medium or hypoxic-conditioned

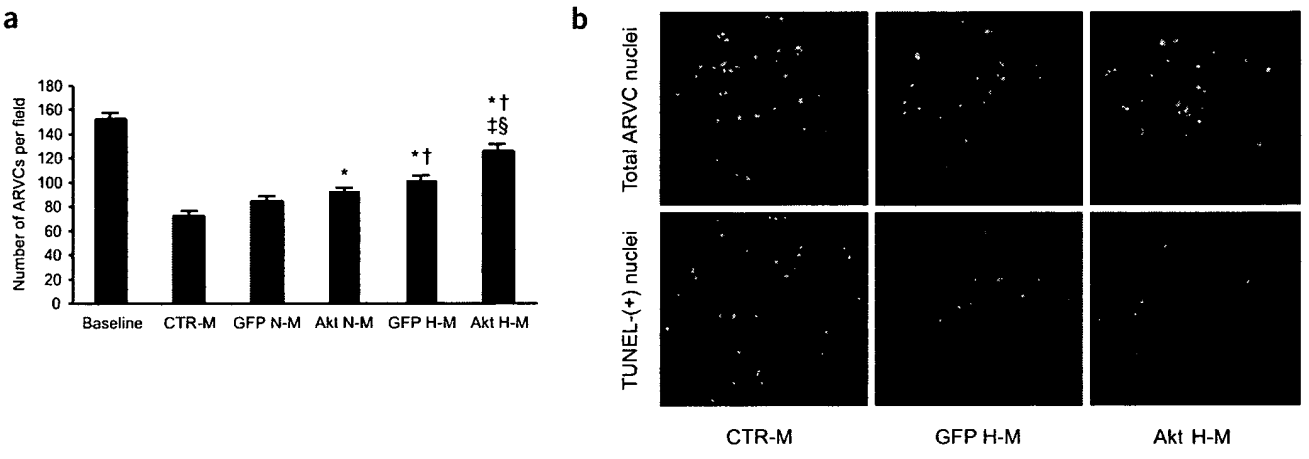

Figure 1 *In vitro* effects of conditioned medium. **(a)** The bar graph summarizes the results of three independent experiments of ARVCs exposed to hypoxia for 24 h in the presence of control (CTR-M) or conditioned medium ($n = 6$ fields counted in 3 different wells for each condition). N-M, normoxic-conditioned medium; H-M, hypoxic-conditioned medium. $^{*}P < 0.05$ versus control medium; $^{\dagger}P < 0.05$ versus GFP normoxic-conditioned medium; $^{\ddagger}P < 0.05$ versus Akt normoxic-conditioned medium; $^{\S}P < 0.05$ versus GFP hypoxic-conditioned medium. Under all the conditions tested, the total number of ARVCs was significantly lower than at baseline ($P < 0.05$). **(b)** Representative photomicrographs of total (upper panels, propidium iodide staining) and TUNEL-positive (lower panels) ARVCs in the presence of control medium, GFP-MSC hypoxic-conditioned medium and Akt-MSC hypoxic-conditioned medium. There were fewer TUNEL-positive ARVCs in the presence of Akt-MSC hypoxic-conditioned medium compared with the control medium or the GFP-MSC hypoxic-conditioned medium. Magnification, ×40.

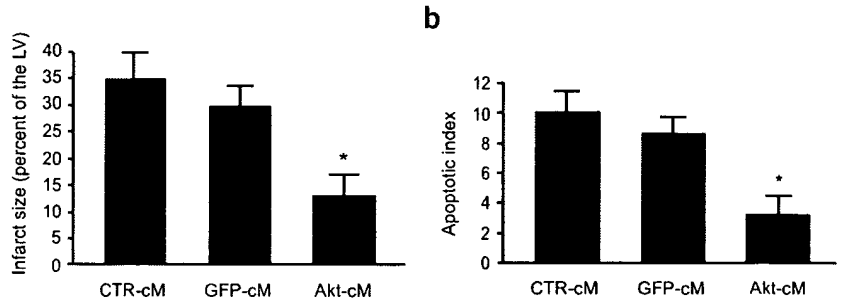

Figure 2 *In vivo* effects of concentrated conditioned medium from MSCs exposed to hypoxia. (**a**) Intramyocardial injection of Akt concentrated medium (Akt-cM) but not GFP concentrated medium (GFP-cM) significantly limited the infarct size 72 h after myocardial infarction ($n = 5$ hearts for each group). CTR-cM, control concentrated medium. LV, left ventricle. (**b**) The apoptotic index was expressed as the proportion of TUNEL-positive cardiomyocyte nuclei from the total number of cardiomyocyte nuclei and the cardiomyocyte origin was identified by the presence of myofilaments surrounding the nucleus. *$P < 0.05$ versus control medium and GFP concentrated medium.

medium from GFP-MSCs or Akt-MSCs; the ARVCs were subsequently exposed to hypoxia for 24 h. When maintained in control medium under normoxic conditions for 24 h (baseline), an average of 153.4 ± 2.9 ARVCs were counted per magnified field (**Fig. 1a**). After exposure to 24 h of hypoxia in the presence of control medium, the total number of ARVCs was considerably reduced ($P < 0.05$ versus normoxic conditions; **Fig. 1a**). Exposure to GFP-MSC normoxic-conditioned medium did not significantly change the total number of ARVCs compared with the control medium (**Fig. 1a**). In contrast, the Akt-MSC normoxic-conditioned medium led to an increase in the number of ARVCs compared with the control medium ($P < 0.05$; **Fig. 1a**). The cytoprotective effect was most notable when the ARVCs were maintained in conditioned medium from MSCs that were exposed to hypoxia. In the presence of GFP-MSC hypoxic-conditioned medium, the total number of ARVCs was increased both as compared with the control medium ($P < 0.05$) and with the GFP-MSC normoxic-conditioned medium ($P < 0.05$; **Fig. 1a**). But the greatest degree of protection was conferred by the Akt-MSC hypoxic-conditioned medium, with an increase in the number of ARVCs as compared with all the other conditions ($P < 0.05$ versus control medium, GFP-MSC normoxic-conditioned medium, Akt-MSC normoxic-conditioned medium and GFP-MSC hypoxic-conditioned medium; **Fig. 1a**). Because apoptosis has a major role in cell loss in the early phase of acute myocardial infarction[8], we determined whether the MSC-conditioned medium exerted an antiapoptotic effect. The relative number of apoptotic ARVCs exposed to hypoxia for 24 h was quantified by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL; **Fig. 1b**). In the presence of Akt-MSC hypoxic-conditioned medium, the number of TUNEL-positive cells was reduced by 62% and 54% as compared with the control medium ($P < 0.05$) or the GFP-MSC hypoxic-conditioned medium ($P < 0.05$), respectively. Thus, these results strongly support our hypothesis of a paracrine cytoprotective mechanism mediated by biologically active factor(s) secreted by the MSCs.

To further validate the protective properties of the Akt-MSCs, we studied the effect of the conditioned medium *in vivo*, using a rat experimental model of permanent coronary occlusion described previously[9]. All animal procedures were approved by the Harvard Standing Committee on Animals Welfare. On the basis of the *in vitro* results, we elected to use medium only from MSCs exposed to hypoxia. Concentrated medium obtained by ultrafiltration (50-fold concentration) was injected into five different sites in the heart at the infarct border zone 30 min after left coronary occlusion. Hearts were isolated 72 h later to examine infarct size and apoptosis. Infarct size, expressed as a ratio of the left ventricular and septal area, was quantified by triphenyltetrazolium chloride (TTC) staining. Injection of GFP concentrated medium did not result in a statistically significant change, whereas the Akt concentrated medium markedly limited the percentage of infarcted tissue ($P < 0.05$ versus control concentrated medium and GFP concentrated medium; **Fig. 2a**). Finally, to test *in vivo* the antiapoptotic properties of the Akt concentrated medium, we performed TUNEL labeling on paraffin-embedded sections and quantified the relative number of positive cardiomyocytes. The apoptotic index was not significantly reduced in the GFP concentrated medium group compared with the control concentrated medium group (**Fig. 2b**). In contrast, a marked antiapoptotic effect was observed after the injection of Akt concentrated medium, yielding a 69% reduction of TUNEL-positive cardiomyocytes as compared with the control concentrated medium group ($P < 0.05$) and a 63% reduction as compared with the GFP concentrated medium group ($P < 0.05$; **Fig. 2b**).

In conclusion, we provide evidence that, especially under hypoxic conditions, genetically modified bone marrow–derived mesenchymal stem cells overexpressing the *Akt1* gene release paracrine factor(s) that exert cytoprotective effects on cardiomyocytes exposed to hypoxia. Notably, we show that myocardial protection can also be achieved *in vivo* by Akt concentrated medium injection after acute infarction. Thus, we propose that a substantial portion of the salutary effects of Akt-MSCs is attributable to protection of ischemic myocardium instead of *de novo* regeneration by cardiomyogenic differentiation of the donor cells. Taken together, our data support a 'paracrine hypothesis' of stem cell action in tissue protection and repair. These findings would suggest that the isolation of the secreted factor(s) may have important therapeutic application for the prevention of ischemic tissue damage.

ACKNOWLEDGMENTS

This work was supported in part by US National Institutes of Health Grants HL 72010, HL73219, HL 58516, HL 35610 (to V.J.D.) and HL 5320, HL 63985 (J.S.I). M. Gnecchi was partially supported by a research award from the University of Pavia and IRCCS Policlinico San Matteo, Pavia, Italy.

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